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Distinct Growth Factor Families Are Recruited in Unique Spatiotemporal Domains during Long-Term Memory Formation in *Aplysia californica*

Highlights

- Trials 1 and 2 recruit synaptic TrkB and somatic TGF-βr-II signaling, respectively
- TrkB and TGF-βr-II act independently to regulate discrete phases of MAPK activation
- TrkB and TGF-βr-II act synergistically to regulate gene expression (*apc/ebp)*
- Both trial 1 TrkB and trial 2 TGF-βr-II signaling are required for LTM formation

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In Brief

Kopec et al. show that distinct growth factor families (TrkB and TGFrβ-II signaling) are recruited in unique spatial and temporal domains to regulate MAPK activation, gene expression, and the induction of long-term memory in *Aplysia*.

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Distinct Growth Factor Families Are Recruited in Unique Spatiotemporal Domains during Long-Term Memory Formation in *Aplysia californica*

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SUMMARY

Several growth factors (GFs) have been implicated in long-term memory (LTM), but no single GF can support all of the plastic changes that occur during memory formation. Because GFs engage highly convergent signaling cascades that often mediate similar functional outcomes, the relative contribution of any particular GF to LTM is difficult to ascertain. To explore this question, we determined the unique contribution of distinct GF families (signaling via TrkB and TGF-βr-II) to LTM formation in *Aplysia*. We demonstrate that TrkB and TGF-βr-II signaling are differentially recruited during two-trial training in both time (by trial 1 or 2, respectively) and space (in distinct subcellular compartments). These GFs independently regulate MAPK activation and synergistically regulate gene expression. We also show that trial 1 TrkB and trial 2 TGF-βr-II signaling are required for LTM formation. These data support the view that GFs engaged in LTM formation are interactive components of a complex molecular network.

INTRODUCTION

Beginning with the pioneering discoveries of Levi-Montalcini, Cohen, and colleagues (Cohen et al., 1954), it is now appreciated that growth factors (GFs) are secreted molecules that bind membrane-associated extracellular receptors, thereby activating intracellular signaling cascades that ultimately mediate developmental plasticity. In recent years, it has also become clear that many of the GFs engaged during development are re-engaged in the adult to support neuronal plasticity involved in memory formation.

Memory can exist in a wide range of temporal domains that can be distinguished by the molecular mechanisms engaged in their induction and maintenance. For example, short-term memory (STM) is mediated by post-translational modifications and lasts minutes to hours, whereas long-term memory (LTM) requires both translation and transcription and can last for several days or longer (Bailey et al., 1996; Castellucci et al., 1989). Intriguingly, while a variety of GFs have been implicated in learning and memory processes, no individual GF can, by itself, support all of the plastic changes that occur during memory formation (Kopec and Carew, 2013). Moreover, there is considerable overlap in the roles different GFs play as critical mediators of plasticity, and their effects are exerted by engaging highly convergent molecular signaling cascades (Huang and Reichardt, 2003; Massagué, 2000). Therefore, the relative contribution of each individual GF to LTM formation is difficult to determine.

Aplysia californica is a powerful model system for studying the molecular mechanisms underlying memory formation (Kandel, 2001). The defensive withdrawal reflexes are mediated in large measure by sensory neuron (SN)-motor neuron (MN) circuits (Marinesco et al., 2006; Philips et al., 2011; Sutton et al., 2001; Walters et al., 1983). LTM for sensitization of the withdrawal reflexes, or its underlying cellular correlate, long-term facilitation (LTF) of SN-MN synapses, can be studied by delivering behavioral training with tail shocks (TSs) or analog training in dissected ganglion preparations with tail nerve shocks (TNSs). Serotonin (5HT) is released within the SN-MN circuits during training (Marinesco and Carew, 2002; Marinesco et al., 2006; Philips et al., 2011), which induces mitogen-activated protein kinase (MAPK) activation and gene expression, both of which are required for LTM formation (Philips et al., 2007, 2013b; Sharma et al., 2003; Sutton et al., 2001). In addition, LTM, LTF, and MAPK activation in *Aplysia* requires signaling from two distinct GF families: tropomyosin-related kinase B (TrkB) signaling, a tyrosine kinase that is the high-affinity receptor for brain derived neurotrophic factor (BDNF), and transforming growth factor β receptor II (TGF-βr-II) signaling, a serine-threonine kinase that is the receptor for TGF-β ligands (Chin et al., 2002; Kassabov et al., 2013; Sharma et al., 2006; Zhang et al., 1997). Furthermore, incubation of pleural-pedal ganglia or SN-MN co-cultures with mammalian GF ligands (BDNF and TGF-β) promotes MAPK activation, synaptic strengthening, and LTM (Chin et al., 2002, 2006; Purcell et al., 2003; Zhang et al., 1997). Collectively, these data indicate the existence of endogenous Trk and TGF-β GF signaling cascades and their recruitment during memory formation.

As in other systems, the pattern of training trials is of critical importance for LTM formation in *Aplysia* (Philips et al., 2013a). For example, we found that a novel two-trial training pattern consisting of two TSs spaced by 45 min results in LTM for sensitization of the withdrawal reflexes (Philips et al., 2007, 2013b). The temporal distance between the two trials in this training pattern is highly advantageous, since it permits the analysis of the contribution of molecular signaling recruited by each individual training

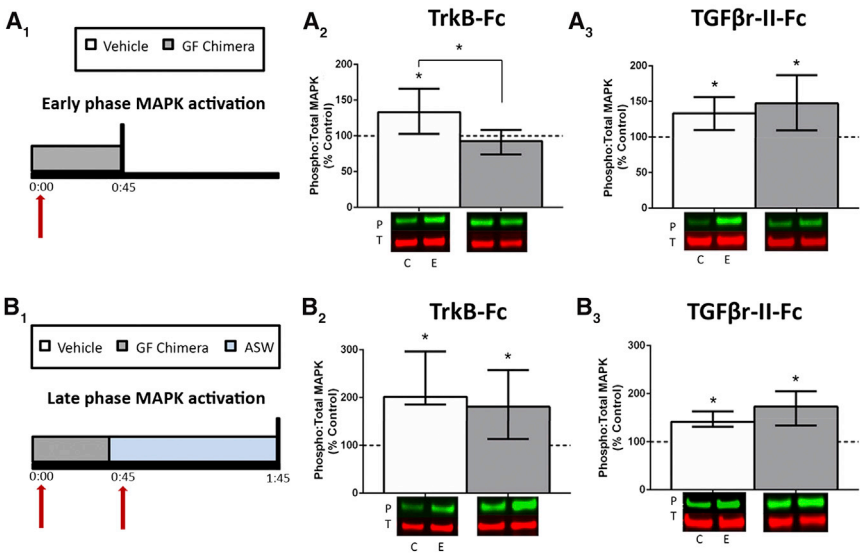

Figure 1. Trial 1 Recruits TrkB but Not TGF-βr-II Signaling for Early- but Not Late-Phase MAPK Activation

(A_1) Experimental paradigm. Dissected ganglia are incubated with GF chimera (gray) or vehicle (white) 10 min prior to trial 1 (time 0:00, red arrow) until SN somata are collected at 0:45 min (vertical black bar).

(A_2) Blocking TrkB signaling during trial 1 significantly disrupts early phase MAPK activation. P, phospho-specific MAPK; T, total MAPK; C, control ganglia; E, experimental ganglia.

(A_3) Blocking TGF-βr-II signaling during trial 1 does not disrupt early-phase MAPK activation.

(B_1) Ganglia are incubated with drug 10 min prior to trial 1 (0:00). Chimera/vehicle is washed out with ASW (blue) 5 min prior to trial 2 (0:45), and SN somata are collected 1 hr post-training (1:45).

(B_2) Blocking TrkB signaling during trial 1 does not disrupt late-phase MAPK activation.

(B_3) Blocking TGF-βr signaling during trial 1 does not disrupt late-phase MAPK activation. Example western blots are displayed below the histograms.

In this and all subsequent figures, data are displayed as median ± IQR, within-group statistics are displayed above histogram bars, and between-group statistics are displayed above the histograms being compared. *p < 0.05, **p < 0.01; n = 6–10.

trial to LTM formation. Moreover, measurement of critical molecular signaling (e.g., MAPK activation and gene expression) as a proxy for LTM formation provides additional tools with which to assess the regulation of individual molecular cascades common to both GF families. Thus, in the present paper, to determine the distinct contributions of different GF families to LTM formation, we asked whether and how TrkB and TGF-βr-II signaling uniquely contribute to MAPK activation, gene expression, and LTM formation induced by two-trial training. We found that trial 1 recruits synaptic TrkB signaling, while trial 2 recruits somatic TGF-βr-II signaling. Furthermore, we found that these GF families act independently to regulate discrete temporal phases of MAPK activation and synergistically to regulate mRNA levels of the transcription factor *apc/ebp*. Finally, these molecular observations gave rise to specific behavioral predictions for LTM formation. Trial 1 TrkB signaling and trial 2 TGF-βr-II signaling should be essential for LTM. Both of these predictions were confirmed by examining the tail-elicited siphon withdrawal reflex.

RESULTS

Trial 1 Recruits TrkB Signaling for Early-Phase MAPK Activation

In response to sensitizing stimuli, MAPK activation in SN somata occurs in two distinct phases: during training and after training. A single training trial induces a transient early phase of MAPK activation in SN somata at 45 min that, in the absence of additional training trials, declines back to baseline by 60 min (Philips et al., 2007; 2013b). However, following repeated-trial training permissive for the induction of LTM, a persistent late phase of MAPK activation is generated in SN somata from 1–3 hr after training (Sharma et al., 2003; Ye et al., 2012). Importantly, both phases of MAPK activation are required for LTM formation (Philips et al., 2013b; J. Shobe et al., 2005, Soc. Neurosci., abstract). As a first step in characterizing the role of distinct GF families during LTM formation, we examined the necessity for TrkB and TGF-βr-II signaling upstream of the early and late phases of MAPK activation.

We first asked whether trial 1 of two-trial analog training (TNSs) recruits GF signaling required for either phase of MAPK activation. To block GF signaling, we utilized TrkB and TGF-βr-II chimeras, which sequester endogenous GF ligands and, thereby, inhibit GF signaling. These chimeras have been used previously to study GF signaling in *Aplysia* (Sharma et al., 2006; Zhang et al., 1997). Trial 1 was delivered to dissected ganglion preparations in the presence of GF chimeras (TrkB-Fc chimera and TGF-βr-II-Fc chimera, R&D Systems, 5μg/ml) or vehicle (0.1% BSA in PBS), and SN somata were collected at 45 min (Figure 1A_1). Consistent with previous reports (Philips et al., 2007; 2013b), there was significant early-phase MAPK activation at 45 min in the TrkB-Fc vehicle (Figure 1A_2; median ± interquartile range (IQR), 133.0% ± 63.6%; Wilcoxon matched-pairs signed rank (within group), $W = 24$; $p < 0.05$; $n = 7$) and TGF-βr-II-Fc vehicle (Figure 1A_3; 133.3% ± 46.2%, $W = 34$, $p < 0.05$; $n = 7$) groups relative to within-animal control levels (see Experimental Procedures). Moreover, this early-phase MAPK activation was significantly attenuated by the TrkB-Fc chimera (Figure 1A_2; 92.7% ± 34.0%; n = 6; Mann-Whitney *U* test (between groups), $U = 5$; $p < 0.05$) but was not disrupted by the TGF-βr-II-Fc chimera (Figure 1A_3; 147.2% ± 77.4%, $W = 34$, $p < 0.05$, n = 8, 1 outlier). These data indicate that trial 1 induces the release of a TrkB ligand that activates early-phase MAPK in SN somata, whereas TGF-βr-II signaling is not engaged by trial 1 for early-phase MAPK activation.

GF Signaling Recruited by Trial 1 Is Not Required for Late-Phase MAPK Activation

Given the results so far, trial 1 could still engage TGF-βr-II signaling to mediate late-phase MAPK activation. Similarly,

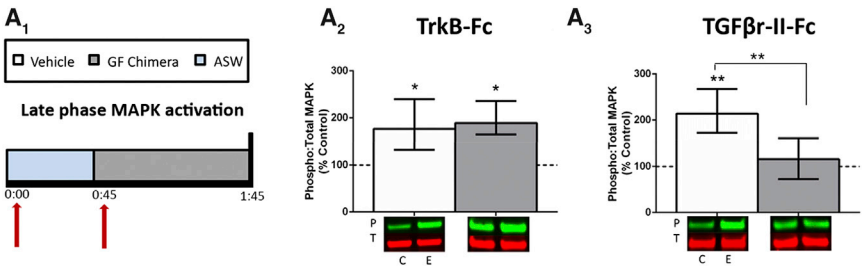

Figure 2. Trial 2 Recruits TGF-βr-II but Not TrkB Signaling for Late-Phase MAPK Activation

(A_1) Trial 1 (0:00) is delivered in the presence of ASW, and the drug is applied 10 min prior to trial 2 (0:45). SN somata are collected 1 hr post-training (1:45).

(A_2) Blocking TrkB signaling during trial 2 does not disrupt late-phase MAPK activation.

(A_3) Blocking TGF-βr-II signaling during trial 2 significantly disrupts late-phase MAPK activation. *$p < 0.05$, **$p < 0.01$; n = 6–9.

TrkB signaling initiated by trial 1 could be required for both the early and late phases of MAPK activation. To explore these possibilities, we next asked whether GF signaling initiated by trial 1 is required for late-phase MAPK activation. Trial 1 was delivered in the presence of the GF chimera or vehicle, which was washed out prior to trial 2, and SN somata were collected 1 hr post-training (Figure 1B_1). As expected, groups receiving TrkB-Fc vehicle (Figure 1B_2; 201.7% ± 111.2%, $W = 28$, $p < 0.05$, n = 7) and TGF-βr-II-Fc vehicle (Figure 1B_3; 141.3% ± 31.4%, $W = 28$, $p < 0.05$, n = 7, 1 outlier) exhibited significant late-phase MAPK activation. Interestingly, both the TrkB-Fc chimera (Figure 1B_2; 181.0% ± 144.2%, $W = 32$, $p < 0.05$, n = 8) and TGF-βr-II-Fc chimera (Figure 1B_3; 172.9% ± 70.6%, $W = 28$, $p < 0.05$, n = 7, 1 outlier) groups also showed significant late-phase MAPK activation. Taken together, these data suggest that each phase of MAPK activation (trial 1-dependent early phase and trial 2-dependent late phase) is independent, since blocking the early, TrkB-dependent phase has no effect on the subsequent late phase. Additionally, TGF-βr-II signaling, if it is engaged by trial 1, is not required for either phase of MAPK activation.

Trial 2 Recruits TGF-βr-II Signaling for Late-Phase MAPK Activation

Given that trial 1 engages GF signaling required for early-phase MAPK activation, we next asked whether trial 2 engages the same or different GF signaling cascades for late-phase MAPK activation. Two-trial analog training was administered, with trial 2 delivered in the presence of the GF chimera or vehicle, and SN somata were collected 1 hr post-training (Figure 2A_1). Both the TrkB-Fc vehicle (Figure 2A_2; 176.4% ± 107.6%, $W = 26$, $p < 0.05$, n = 7) and TGF-βr-II-Fc vehicle (Figure 2A_3; 213.8% ± 95.0%, $W = 45$, $p < 0.01$, n = 9) groups showed significant late-phase MAPK activation. Moreover, the TrkB-Fc chimera, during trial 2, did not disrupt late-phase MAPK activation (Figure 2A_2; 189.1% ± 71.4%, $W = 21$, $p < 0.05$, n = 6). In contrast, the TGF-βr-II-Fc chimera, during trial 2, did disrupt late-phase MAPK activation (Figure 2A_3; 115.8% ± 87.9%, n = 9). A Kruskal-Wallis test of drug treatment (TGF-βr-II-Fc chimera or vehicle during trial 1 or during trial 2) indicated a difference among groups ($H = 8.6$, degrees of freedom (df) = 3, $p < 0.05$), and subsequent planned comparisons revealed a significant difference between the TGF-βr-II-Fc chimera and vehicle groups when the drug was applied during trial 2 ($U = 7$, $p < 0.01$). Collectively, these data indicate that there is a temporal dissociation of GF family recruitment during two-trial analog training. Trial 1 preferentially engages TrkB signaling to mediate early-phase MAPK activation, whereas trial 2 preferentially engages TGF-βr-II signaling to mediate late-phase MAPK activation.

TrkB Signaling Is Initiated at the Synapse

Having identified a temporal dissociation for the requirement of different GF families during two-trial analog training, we next examined the spatial profile for signaling by each GF family during its unique temporal phase of training using a split-bath preparation, which permits manipulation of GF signaling in the SN-MN circuit independently in either a somatic compartment, containing SN somata, or a synaptic compartment, containing the SN-MN synapses and MN somata (Sherff and Carew 1999).

First, we asked whether trial 1 TrkB signaling required for early phase MAPK activation is initiated in the somatic or synaptic compartment. We found that, when TrkB signaling is blocked in the somatic compartment, early-phase MAPK activation is not affected (Figure 3A, left; TrkB-Fc vehicle, 134.4% ± 48.2%, $W = 45$, $p < 0.01$, n = 9, 1 outlier; TrkB-Fc chimera, 128.5% ± 39.0%, $W = 36$, $p < 0.01$, n = 8, 1 outlier). However, when TrkB signaling is blocked in the synaptic compartment, early-phase MAPK activation is disrupted significantly (Figure 3A, right; TrkB-Fc vehicle, 147.7% ± 24.7%, $W = 26$, $p < 0.05$, n = 7; TrkB-Fc chimera, 106.2% ± 60.9%, n = 6). A Kruskal-Wallis analysis indicated that there was a difference among groups ($H = 8.3$, df = 3, $p < 0.05$), and subsequent planned comparisons revealed a significant difference between synaptic TrkB-Fc chimera and vehicle treatment on early-phase MAPK activation ($U = 4$, $p < 0.05$). These data indicate that trial 1 induces the release of a TrkB ligand at SN-MN synapses, which mediates early-phase MAPK activation in SN somata. Intriguingly, these data also suggest the requirement of a retrogradely transported intracellular signal arising from the synapse to regulate somatic molecular events (see Discussion).

TGF-βr-II Signaling Is Initiated at the Soma

We next examined the site of trial 2 TGF-βr-II signaling required for late-phase MAPK activation in SN somata. In contrast to TrkB (Figure 3A), blocking TGF-βr-II signaling in the synaptic compartment did not disrupt late-phase MAPK activation (Figure 3B, right; TGF-βr-II-Fc vehicle, 145.7% ± 43.7%, $W = 21$, $p < 0.05$, n = 6, 1 outlier; TGF-βr-II-Fc chimera: 169.0% ± 60.6%, $W = 21$, $p < 0.05$, n = 6). However, blocking TGF-βr-II signaling in the somatic compartment completely blocked late-phase MAPK activation (Figure 3B, left; TGF-βr-II-Fc vehicle, 148.5% ± 55.5%, $W = 28$, $p < 0.05$, n = 7, 1 outlier; TGF-βr-II-Fc chimera, 102.2% ± 40.7%, n = 6). A Kruskal-Wallis analysis

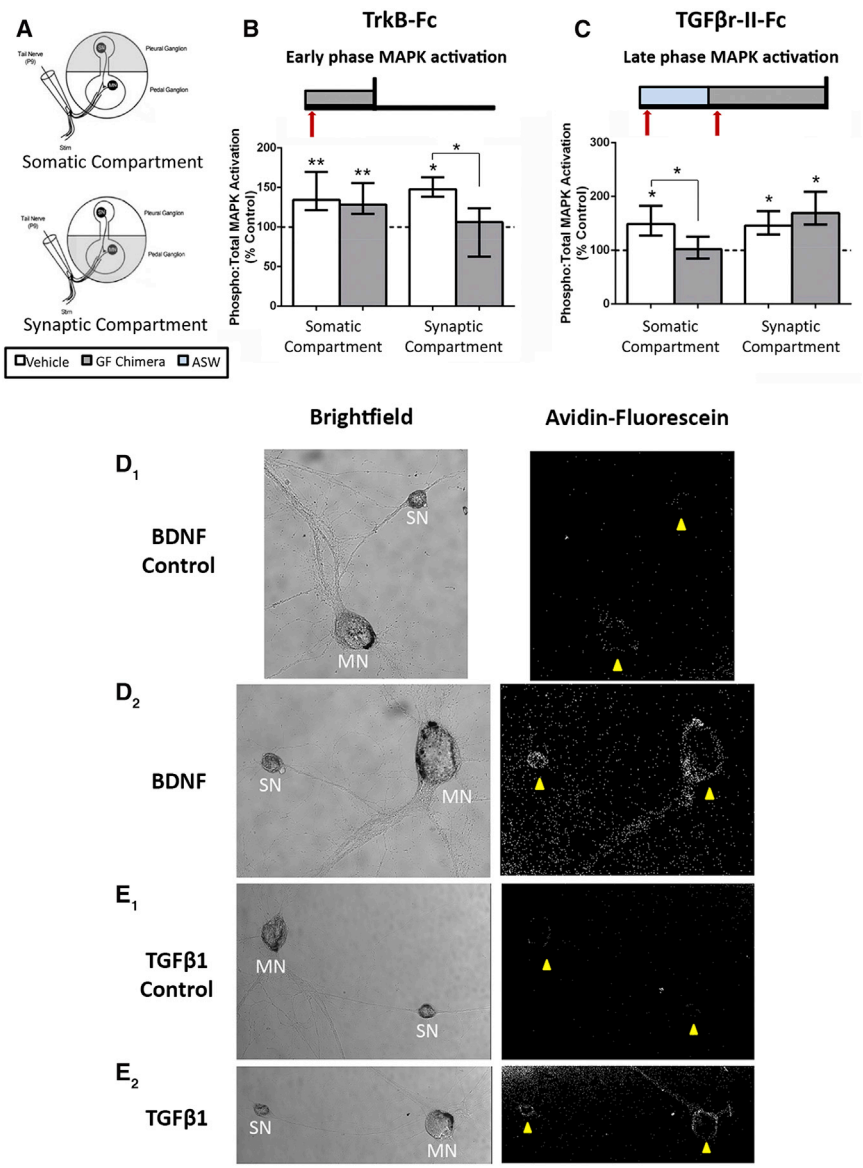

Figure 3. Spatial Dissociation of GF Engagement: TrkB Signaling Is Initiated at the Synapse, whereas TGF-βr-II Signaling Is Initiated at the Soma

(A) To determine where GF signaling is initiated (soma or synapse), a barrier was built between the SN somata and SN-MN synapses, and drug was applied to one compartment. The critical phase of MAPK activation shown to be dependent upon each GF family is used as a readout (Figures 1 and 2; early phase requires TrkB signaling, and late phase requires TGF-βr-II signaling).

(B) Blocking TrkB signaling during trial 1 in the synaptic but not somatic compartment significantly disrupts early-phase MAPK activation.

(C) Blocking TGF-βr-II signaling during trial 2 in the somatic but not synaptic compartment significantly disrupts late-phase MAPK activation. *$p < 0.05$, **$p < 0.01$; $n = 6–9$.

(D and E) Receptor localization for GF ligands was assessed in SN-MN co-cultures stimulated with control biotinylated protein or biotinylated GF ligand, followed by avidin-fluorescein to visualize binding. Confocal images reveal that SNs and MNs bind both BDNF (D_2) and TGFβ1 (E_2) but not control protein (D_1 and E_1). Yellow triangles indicate the location of cell bodies in the avidin-fluorescein images.

indicated that there was a significant difference among groups ($H = 9.3$, df = 3, $p < 0.05$), and subsequent planned comparisons revealed a significant difference between somatic TGF-βr-II-Fc chimera and vehicle treatment on late-phase MAPK activation ($U = 5$, $p < 0.05$). These data indicate that trial 2 induces the release of a TGF-βr-II ligand at SN somata. Therefore, our results reveal a double dissociation in space and time. Trial 1 recruits TrkB signaling at the synapse, and trial 2 recruits TGF-βr-II signaling at the soma. Interestingly, incubation of SN-MN co-culture with biotinylated human recombinant TrkB and TGF-βr-II ligands (BDNF and TGF-β1, respectively) reveals the potential for human ligands to bind receptors on both SNs and MNs (Figures 3D and 3E). Taken together, these data indicate that although there may be receptors for both families of GFs on both the pre- and post-synaptic components of this circuit, their engagement is spatially restricted during two-trial analog training.

Both Trial 1 and Trial 2 Increase *apc/ebp* mRNA Expression

Thus far, our results demonstrate that distinct GF families regulate discrete temporal phases of MAPK activation. Since transcription is uniquely required for long-term forms of plasticity and memory (Bailey et al., 1996), we next asked whether GF-initiated signaling was upstream of learning-related gene expression. We focused our attention on three genes: *Aplysia* CCAAT enhancer-binding protein (ApC/EBP), which is an immediate early gene and transcription factor (Alberini et al., 1994); *Aplysia* ubiquitin C-terminal hydrolase (ApUCH), which is an immediate early gene and associates with the proteasome to increase protein degradation (Hegde et al., 1997); and *Aplysia* kinesin heavy chain 1 (ApKHC1), which is a component of the anterograde motor protein kinesin that transports cargo proteins and mRNAs from the soma to the synapse (Puthanveettil et al., 2008). Importantly, all of these genes have been demonstrated to be regulated by sensitizing stimuli in *Aplysia* and are required for LTF (Alberini et al., 1994; Hegde et al., 1997; Puthanveettil et al., 2008). We first determined when mRNA expression of these learning-related genes is increased (by trial 1 and/or by trial 2), and then asked whether GF signaling is required for increased expression.

SN somata were collected either 45 min after trial 1, 60 min after trial 1, or 15 min after trial 2. RNA was isolated for cDNA synthesis, and then the expression of *apc/ebp*, *apuch*, and *apkhc1*

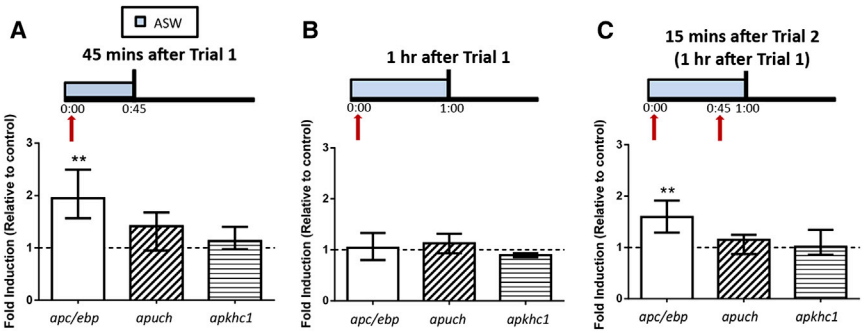

Figure 4. Both Trial 1 and Trial 2 Increase *apc/ebp* mRNA Expression

(A) Trial 1 (0:00) is delivered, and SN somata are collected at 0:45 min. *apc/ebp*, but not *apuch* or *apkhc1* expression, is increased significantly by trial 1.

(B) SN somata are collected 1 hr (1:00) after trial 1. *apc/ebp* expression has returned to baseline 1 hr after trial 1.

(C) SN somata are collected 15 min after trial 2 (1:00). *apc/ebp*, but not *apuch* or *apkhc1* expression, is increased significantly by trial 2.

**p < 0.01, n = 6–10.

mRNA levels was analyzed using quantitative PCR (normalized to the ubiquitous housekeeping gene *apgapdh*; see Experimental Procedures). Kruskal-Wallis analyses indicated that there was a significant difference in gene expression 45 min after trial 1 ($H = 6.9$, df = 2, $p < 0.05$) and 15 min after trial 2 ($H = 9.2$, df = 2, $p < 0.05$) but not 60 min after trial 1 ($H = 3.5$, df = 2, $p = 0.2$). Subsequent planned comparisons revealed that *apc/ebp* mRNA expression is increased significantly 45 min after trial 1 (Figure 4A; 1.9 ± 0.9, $W = -51$, $p < 0.01$, n = 10) and, in the absence of trial 2, returns to control levels 60 min after trial 1 (Figures 4B; 1.0 ± 0.5, n = 6), which is in agreement with our prior work (Philips et al., 2013b). However, when Trial 2 is delivered, *apc/ebp* expression is increased significantly at an equivalent time point (15 min after trial 2; Figure 4C; 1.6 ± 0.6, $W = -36$, $p < 0.01$, n = 8). In contrast, *apuch* and *apkhc1* mRNA levels were not regulated at any time point assessed (Figure 4). Since *apuch* and *apkhc1* expression levels have been reported to be regulated by sensitizing stimuli in *Aplysia* (Hegde et al., 1997; Mohamed et al., 2005; Puthanveettil et al., 2008), these data suggest either that *apuch* and *apkhc1* expression is regulated at different points in time or that different training patterns that result in the same functional outcome (e.g., LTM or LTF) may recruit distinct gene expression profiles. Taken together, these results indicate that increased *apc/ebp* expression is dependent upon both trial 1 and trial 2.

Trial 1-Dependent *apc/ebp* Expression Requires TrkB Signaling during Trial 1

To examine the requirement of GF signaling during trial 1 for trial 1-dependent *apc/ebp* expression, we performed experiments in the presence of the GF chimera or vehicle (Figure 5A_1). *apc/ebp* expression was elevated significantly 45 min after trial 1 in the presence of TrkB-Fc vehicle (Figure 5A_2; 2.1 ± 0.9, $W = -28$, $p < 0.05$, n = 7) and TGF-βr-II-Fc vehicle (Figure 5A_3; 1.5 ± 1.6, $W = -26$, $p < 0.05$, n = 7). However, trial 1-dependent *apc/ebp* expression was disrupted significantly by treatment with the TrkB-Fc chimera (Figure 5A_2; 1.1 ± 0.7, n = 6; $U = 3$, $p < 0.01$) but not the TGF-βr-II-Fc chimera (Figure 5A_3; 1.7 ± 1.0, $W = -28$, $p < 0.05$, n = 7). *apuch* and *apkhc1* mRNA levels were analyzed by quantitative PCR (qPCR) in parallel with *apc/ebp* (Figure 5) and confirmed the lack of regulation seen in the initial experiment (Figure 4A). Our results are consistent with our previous findings (Figure 1) and support the hypothesis that TrkB signaling is preferentially engaged by trial 1 to regulate MAPK activation and gene expression.

Trial 2-Dependent *apc/ebp* Expression Requires TGF-βr-II Signaling during Trial 2

We next tested the hypothesis that trial 2 preferentially recruits TGF-βr-II signaling to mediate Trial 2-dependent *apc/ebp* expression. Two-trial analog training was administered, with trial 2 delivered in the presence of GF chimera or vehicle, and SN somata were collected 15 min post-training (Figure 5B_1). In the presence of TrkB-Fc vehicle (Figure 5B_2; 1.7 ± 0.4, $W = -34$, $p < 0.05$, n = 8) and TGF-βr-II-Fc vehicle (Figure 5B_3; 1.5 ± 0.4, $W = -28$, $p < 0.05$, n = 7, 1 outlier) during trial 2, *apc/ebp* expression was increased significantly 15 min post-training. Trial 2-dependent *apc/ebp* expression was disrupted by blocking TGF-βr-II signaling during trial 2 (Figure 5B_3; 1.0 ± 0.3, n = 8) but not TrkB signaling during trial 2 (Figure 5B_2; 1.5 ± 0.7, $W = -26$, $p < 0.05$, n = 7). Furthermore, a Kruskal-Wallis test of drug treatment (TGF-βr-II-Fc chimera or vehicle during trial 1 (Figure 6A_3) or during trial 2) indicated a difference among groups ($H = 9.2$, df = 3, $p < 0.05$), and subsequent planned comparisons revealed a significant difference between TGF-βr-II-Fc chimera and vehicle groups when the drug was applied during trial 2 ($U = 6$, $p < 0.01$). Taken together, these data are consistent with the notion that there are distinct signaling profiles for each GF family. Trial 1 specifically engages TrkB signaling, and trial 2 specifically engages TGF-βr-II signaling to support both MAPK activation and *apc/ebp* expression.

Trial 2 TGF-βr-II Signaling Prolongs TrkB-Dependent *apc/ebp* Expression Established by Trial 1

Since *apc/ebp* expression is regulated by both TrkB (trial 1-dependent; Figure 5A_2) and TGF-βr-II signaling (trial 2-dependent; Figure 5B_3), we next sought to explore the regulation of the same transcript by both GF families. There are two general possibilities that could account for the results shown in Figure 5: trial 1 increases *apc/ebp* expression, and trial 2 prolongs the expression of these mRNAs, or trial 1 and trial 2 both independently increase *apc/ebp* expression. In the former case, if trial 1 increases *apc/ebp* expression, which, in turn, is prolonged by trial 2, then blocking trial 1 TrkB signaling should block trial 2-dependent *apc/ebp* expression because there would be no mRNA for trial 2 to prolong. In contrast, if trial 2 independently increases *apc/ebp* expression, then blocking trial 1 TrkB signaling should have no effect on trial 2-dependent *apc/ebp* expression. Trial 1 was delivered in the presence of the GF chimera or vehicle, which was washed out prior to trial 2, and SN somata were collected 15 min post-training (Figure 6A_1). Blocking TrkB

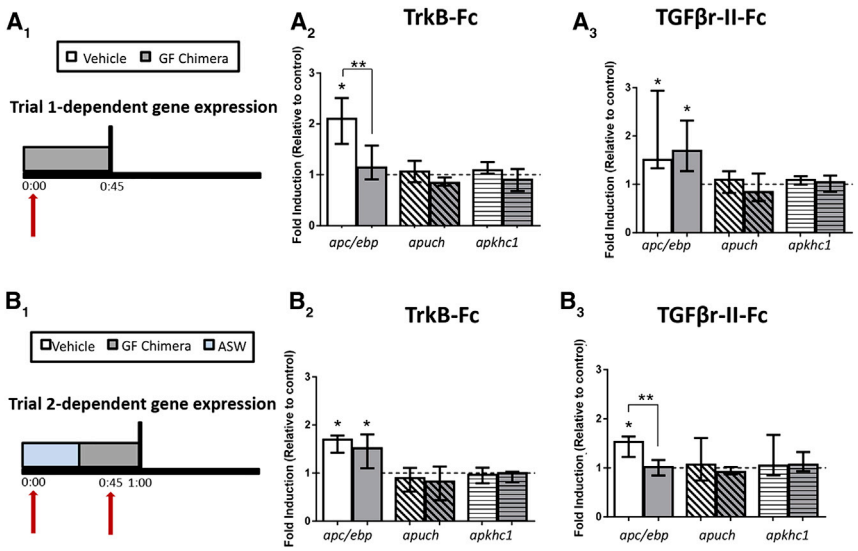

Figure 5. Trial 1-Dependent *apc/ebp* Expression Requires TrkB Signaling during Trial 1, and Trial 2-Dependent *apc/ebp* Expression Requires TGF-βr-II Signaling during Trial 2

(A_1) Ganglia are incubated with drug 20 min prior to trial 1 (0:00), and SN somata are collected at 0:45 min.

(A_2) Blocking TrkB signaling during trial 1 significantly disrupts trial 1-dependent *apc/ebp* expression.

(A_3) Blocking TGF-βr-II signaling during trial 1 does not disrupt trial 1-dependent *apc/ebp* expression.

(B_1) Trial 1 (0:00) is delivered in the presence of ASW, and drug is applied 20 min prior to trial 2 (0:45). SN somata are collected 15 min post-training (1:00).

(B_2) Blocking TrkB signaling during trial 2 does not disrupt trial 2-dependent *apc/ebp* expression.

(B_3) Blocking TGF-βr-II signaling during trial 2 significantly disrupts trial 2-dependent *apc/ebp* expression.

*p < 0.05, **p < 0.01; n = 6–8.

signaling during trial 1 did, in fact, disrupt trial 2-dependent *apc/ebp* expression (Figure 6A_2; TrkB-Fc vehicle, 1.4 ± 0.5, $W = -43$, $p < 0.01$, n = 9; TrkB-Fc chimera, 1.0 ± 0.3, n = 8), while blocking TGFβr-II signaling during trial 1 had no effect (Figure 6A_3; TGF-βr-II-Fc vehicle, 1.3 ± 0.1, $W = -26$, $p < 0.05$, n = 7, 1 outlier; TGF-βr-II-Fc chimera, 1.3 ± 0.4, $W = -21$, $p < 0.05$, n = 6). A Kruskal-Wallis test of drug treatment (TrkB-Fc chimera or vehicle during trial 1 or trial 2) indicated a difference among groups ($H = 9.3$, df = 3, $p < 0.05$), and subsequent planned comparisons revealed a significant difference between the TrkB-Fc chimera and vehicle groups when the drug was applied during trial 1 ($U = 10$, $p < 0.05$). These data therefore support the hypothesis that trial 2 TGF-βr-II signaling interacts with the TrkB signaling cascade to prolong *apc/ebp* expression established by trial 1.

Trial 1 TrkB Signaling and Trial 2 TGF-βr-II Signaling Are Required for LTM for Sensitization

The molecular observations we obtained so far provide clear predictions for the role of GFs in the induction of LTM for sensitization in *Aplysia*. Specifically, our molecular data predict that TrkB signaling should be recruited by trial 1 for LTM formation, and TGF-βr-II signaling should be recruited by trial 2 for LTM formation. We directly tested these hypotheses in a final set of behavioral experiments examining LTM for sensitization of the defensive tail-elicited siphon withdrawal reflex (T-SWR). We used the T-SWR semi-intact preparation (Figure 7A; Philips et al., 2007; Sutton et al., 2001) to manipulate the molecular environment of the CNS while directly measuring withdrawal responses (see Experimental Procedures).

To test the hypothesis that TrkB signaling is recruited by trial 1 for LTM formation, we exposed the CNS to the TrkB-Fc chimera or vehicle during trial 1, which was washed out prior to trial 2. In the presence of TrkB-Fc vehicle, significant LTM for sensitization of the T-SWR was observed (Figure 7B; 154.0% ± 68.4%, $W = 21$, $p < 0.05$, n = 6). In contrast, the induction of LTM was disrupted significantly when TrkB signaling was blocked during trial 1 (Figure 7B; 103.9% ± 6.5%, n = 6, 1 outlier; $U = 0$, $p < 0.01$).

To test the hypothesis that TGF-βr-II signaling is recruited by trial 2 for LTM formation, we administered two-trial training with the CNS exposed to the TGF-βr-II chimera or vehicle during trial 2. In the presence of TGF-βr-II-Fc vehicle, significant LTM for sensitization was induced (Figure 7C; 179.4% ± 59.3%, $W = 21$, $p < 0.05$, n = 6). In contrast, LTM was disrupted significantly when TGF-βr-II signaling was blocked during trial 2 (Figure 7C; 109.4% ± 14.5%, n = 6; $U = 3$, $p < 0.05$). These behavioral data confirm the predictions derived from our molecular observations and support a general model in which trial 1 recruits TrkB signaling and trial 2 recruits TGF-βr-II signaling to mediate molecular changes critical for LTM formation.

DISCUSSION

In this paper, we show that GF signaling via TrkB and TGF-βr-II receptors is highly regulated both temporally (during trial 1 versus during trial 2) and spatially (synapse versus soma) in response to stimuli that induced LTM in *Aplysia*. Collectively, our data support the working model shown in Figure 8. We propose that trial 1 recruits synaptic TrkB signaling to mediate early-phase MAPK activation and increases in *apc/ebp* mRNA expression (Figure 8A). Subsequently, trial 2 recruits somatic TGF-βr-II signaling, which produces two effects. It synergistically interacts with the molecular cascade established by TrkB signaling to prolong *apc/ebp* mRNA expression, and it independently induces late-phase MAPK activation (Figure 8B). Furthermore, the molecular network comprised of trial 1 TrkB-Trial 2 TGF-βr-II is required for the induction of LTM for sensitization of the T-SWR (Figure 7). To our knowledge, these experiments provide the first evidence for the parallel recruitment of distinct GF families by different training trials during LTM formation, the engagement of different GF families in distinct subcellular compartments as a function of training pattern, and the interaction between specific molecular signaling cascades that have been engaged by distinct GF families during LTM formation.

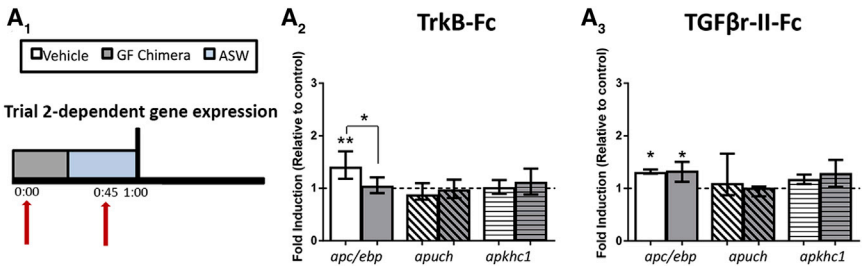

Figure 6. Trial 2 TGF-βr-II Signaling Prolongs *apc/ebp* Expression Established by Trial 1 TrkB Signaling

(A_1) Ganglia are incubated with drug 20 min prior to trial 1 (0:00), which is washed out with ASW 15 min prior to trial 2. SN somata are collected 15 min post-training (1:00).

(A_2) Blocking TrkB signaling during trial 1 significantly disrupts trial 2-dependent *apc/ebp* expression.

(A_3) Blocking TGF-βr-II during trial 1 does not disrupt trial 2-dependent *apc/ebp* expression.

*$p < 0.05$, **$p < 0.01$; n = 6–9.

Temporal Dissociation of GF Signaling during Memory Formation

During neuronal development, GF signaling is tightly controlled in time and space to create a specific, stereotyped neural circuit (Cohen-Cory et al., 2010; Heerssen and Segal, 2002; Massagué, 2000). Our data are consistent with emerging evidence indicating that similar temporal and spatial regulation of GF signaling occurs during memory formation (Kopec and Carew, 2013).

Our core observations summarized in Figure 8 raise a significant question. Why does Trial 1 preferentially recruit TrkB signaling at the synapse, while trial 2 preferentially recruits TGF-βr-II signaling at the soma? The mRNA for BDNF (the mammalian ligand for TrkB) has been shown to be targeted to and stored at synapses and is rapidly translated and released in response to activity-dependent stimuli (Kuczewski et al., 2009; Tongiorgi, 2008). BDNF can therefore be considered the "first responder" to learning-related stimuli and, as our data demonstrate, is poised to prime a neural circuit for information storage (Figure 8A). Consistent with this notion, in studies examining memory formation, BDNF-TrkB signaling is often reported to be required either during or shortly after behavioral training (Kuczewski et al., 2009; Tongiorgi, 2008). That said, there is also clear evidence for later phases of BDNF signaling at time points after training (Bambah-Mukku et al., 2014; Bekinschtein et al., 2007, 2008) to support memory persistence.

The preferential engagement of TGF-βr-II signaling by trial 2 (Figure 8B) supports the view that the molecular environment established by trial 1 alone may not be sufficient to support TGF-βr-II signaling. There are at least three possibilities that might account for this observation: there is no TGF-βr-II ligand released by trial 1, there are not sufficient receptors to transduce signaling by TGF-βr-II ligands released during trial 1, and there is a limiting factor in the TGF-β-TGF-βr-II signaling cascade that is not active during trial 1. To distinguish among these possibilities, in future studies it will be critical to determine whether protein and mRNA levels of TGF-βr-II and its ligand are regulated by individual training trials during memory formation. Previous evidence showed that TGF-β can be released in a pro-form that requires extracellular cleavage by metalloproteases and/or integrins to signal via its receptor (Annes et al., 2003). Therefore, the protein or activity levels of proteases could orchestrate the temporal profile in which TGFβ signaling occurs. Liu and colleagues (1997) have characterized an *Aplysia* homolog of the *Drosophila* metalloprotease Tolloid. Interestingly, Tolloid mRNA is upregulated by sensitizing stimuli, indicating that the induction and translation of Tolloid could be a prerequisite to TGF-βr-II signaling, which may, in part, explain the delayed engagement of this GF family.

The data in this paper indicate that GF signaling in temporally distinct profiles mediates discrete temporal phases of MAPK activation induced by learning-related stimuli (Figures 1 and 2). In a wide range of preparations, MAPK is activated by learning-related stimuli both during and after training (Sweatt, 2004). However, whether there are mechanistically distinct phases of activation is not well understood. Our results indicate that, in *Aplysia* SN somata, there are at least two independent phases of MAPK activation: an early phase that can be identified during the inter-trial training interval and a late (persistent) phase that is evident only after the second trial. The induction of persistent MAPK activation after training as well as a requirement for MAPK activation for long-lasting behavioral and cellular plasticity are well described (for a review, see Thomas and Huganir, 2004). However, inter-trial MAPK activation has only recently been characterized (Ajay and Bhalla, 2004; Pagani et al., 2009; Philips et al., 2007, 2013b). For example, we recently reported that an early phase of activated MAPK translocates to the nucleus, activates the CREB kinase p90rsk, regulates *apc/ebp* expression, and is required for two-trial LTM (Philips et al., 2013b). The functional significance of late phases of MAPK activation that are required for LTM in *Aplysia* (J. Shobe et al., 2005, Soc. Neurosci., abstract) remain to be elucidated, and the relative contribution of distinct phases of MAPK activation across trials during memory induction remains an open question.

Spatial Dissociation of GF Signaling during Memory Formation

Our data show that, in addition to a temporal dissociation, signaling from different GF families is also recruited in spatially distinct subcellular compartments. Although human recombinant BDNF and TGF-β1 bound to both pre- and post-synaptic elements within *Aplysia* co-culture (Figures 3D and 3E), there appears to be a spatial dissociation of the functional engagement of GF signaling. Specifically, TrkB signaling is initiated at the SN-MN synapse, while TGF-βr-II signaling is initiated at the SN somata (Figures 3B and 3C). Previous reports have indicated the localization of neurotrophin-like ligands and Trk-like receptors in both the SN and MN (Kassabov et al., 2013; Ormond et al., 2004; Pu et al., 2014). Taken together, these data suggest that GF signaling via Trk-like receptors may occur both pre- and post-synaptically. The working model in Figure 8 depicts

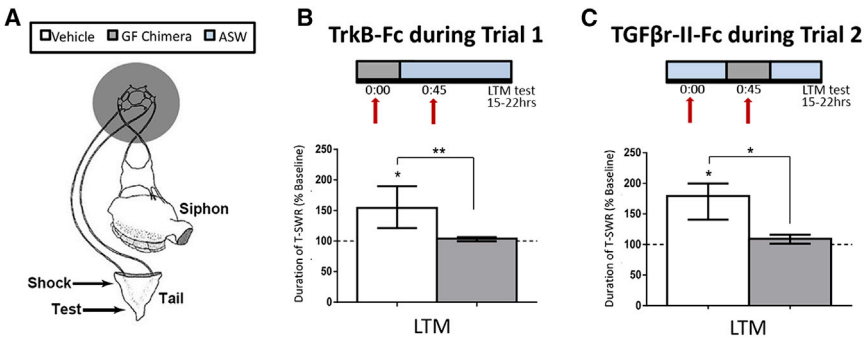

Figure 7. Trial 1 TrkB Signaling and Trial 2 TGF-βr-II Signaling Are Required for Two-Trial LTM for Sensitization of the T-SWR

(A) Semi-intact preparation. Two-trial behavioral training is administered to the training site, and drug is applied to the isolated CNS chamber in the experimental paradigms shown above the data histograms. LTM is measured by stimulating the test site 15–22 hr after training and measuring the T-SWR.

(B) Blocking TrkB signaling during trial 1 significantly disrupts LTM formation.

(C) Blocking TGF-βr-II signaling during trial 2 significantly disrupts LTM formation.

*$p < 0.05$, **$p < 0.01$; $n = 6$.

TrkB- and TGF-βr-II-like receptors localized to the pre-synaptic SN to emphasize that the molecular events regulated by these GF families are occurring in SNs. However, post-synaptic TrkB-like receptors could also modulate pre-synaptic molecular events via a *trans*-synaptic retrograde signaling mechanism. Interestingly, one such retrograde messenger, nitric oxide, has been shown to be induced in hippocampal neurons in a TrkB-dependent manner (Kolarow et al., 2014).

Importantly, the somatic and synaptic compartments include important circuit components in addition to the SN somata and SN-MN synapses (Cleary et al., 1995; Marinesco et al., 2004). Although the SN-MN synapse within the withdrawal circuit is often sufficient to capture essential features of the plasticity-underlying behavior in *Aplysia*, these other components of the circuit may indeed play important roles in sensitization memory (Cleary et al., 1995; White et al., 1993). It is important to note that the post-synaptic MN has an important role in plasticity and memory in *Aplysia* (Glanzman, 2008), and determining whether and how GF signaling is engaged in the MN will be of considerable interest. Therefore, although our data demonstrate the importance of synaptic TrkB signaling and somatic TGF-βr-II signaling and the capability for both ligands to bind both pre- and post-synaptic elements in the withdrawal circuit, the cellular source of these ligands remains to be elucidated.

One potential advantage for the spatial dissociation of GF family engagement could be to mediate different functional outcomes depending on the spatial location of the signaling in the neuron. For instance, synaptic TrkB signaling could be poised to quickly modulate local protein synthesis, while somatic TGFβr-II signaling could have faster or easier access to the nucleus for transcriptional regulation. Our data indicate that, despite GF signaling arising from spatially disparate locations in the neural circuit, both TrkB and TGF-βr-II signaling interact to regulate the same signaling molecule in the same subcellular compartment: MAPK in SN somata. Importantly, whether the same pool of MAPK is being regulated in both cases remains to be clarified. In principle, there could be distinct pools of MAPK activated by each GF, which could allow each pool of MAPK to mediate different functional outcomes.

The requirement of synaptic TrkB signaling for subsequent somatic events suggests the involvement of a retrogradely transported intracellular signal (Figure 8A). Interestingly, Jeanneteau et al. (2010) have provided evidence consistent with this idea. They found that, in primary cortical cultures, stimulation of distal axons with either BDNF or with depolarizing stimuli was sufficient to induce the transient induction of the gene for a MAPK phosphatase, *mkp-1*, an effect that requires MEK activity. These observations, which are similar to ours, suggest that a synaptic, TrkB-dependent, retrogradely transported, intracellular signal resulting in somatic MAPK activation and gene expression may be a conserved first step in the response to memory-inducing stimuli. Two reasonable candidates for a possible retrogradely transported intracellular signal are a signaling endosome and/or MAPK itself. Considering the first candidate, it is known that, during development, GFs binding to Trk receptors cause the internalization of the ligand-receptor complex, giving rise to a signaling endosome. The tyrosine kinase domain of Trk in this endosome is exposed to the intracellular environment and phosphorylates targets as the endosome moves to the soma (Heerssen and Segal, 2002). Considering the second idea, MAPK itself is phosphorylated at the synapse and could travel to the soma, where it translocates into the nucleus to regulate transcription (Philips et al., 2013b). Indeed, phosphorylated MAPK has been shown to be transported to the soma via importin-mediated nuclear transport in response to axonal injury (Perlson et al., 2006), and, interestingly, importin-mediated transport is required for LTF in *Aplysia* (Thompson et al., 2004).

RNA Regulation during Memory Formation

Another novel feature of our results is the demonstration of a synergistic interaction between distinct GF families recruited during memory induction. Specifically, trial 1 TrkB signaling increases the expression of *apc/ebp* mRNA, and trial 2 TGF-βr-II signaling prolongs the expression of these mRNAs (Figure 6). These data highlight two main points: distinct GF families synergistically promote the expression of *ap/cebp*, and TGF-βr-II signaling mediates the post-transcriptional regulation of *apc/ebp* mRNAs. An important question now will be to determine how TGF-βr-II signaling mediates mRNA regulation.

Gene induction downstream of the transcription factor C/EBP is a highly conserved requirement for LTM and long-lasting plasticity (Alberini, 2009). In *Aplysia*, the homolog of C/EBP has a short and long isoform and is most similar to mammalian C/EBPβ (Alberini et al., 1994). When a constitutively expressed transcription factor, ApAF, is phosphorylated by PKA, it dimerizes with ApC/EBP and induces transcription. The constitutive

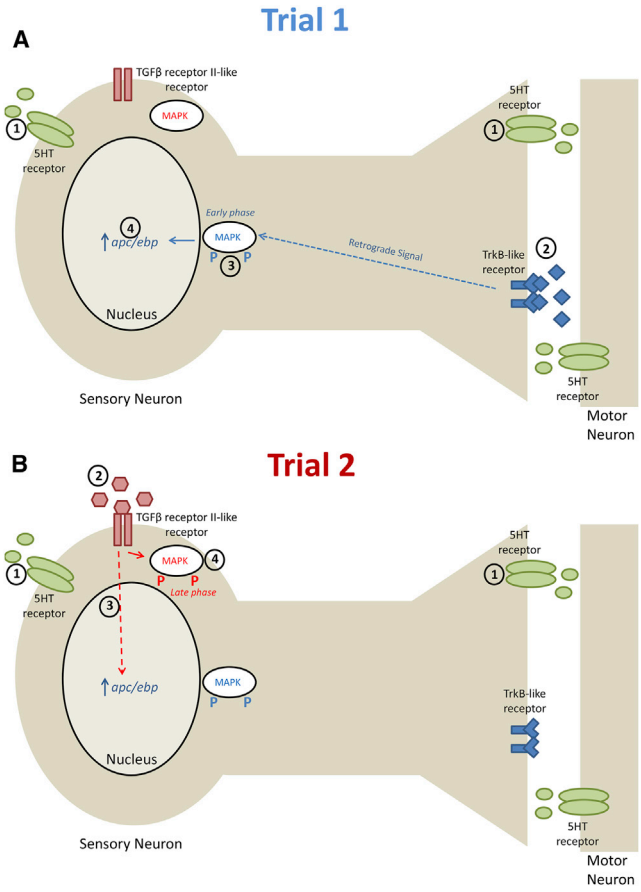

Figure 8. Working Model of GF Signaling in a Molecular Network during Two-Trial LTM Formation

(A) Molecular signaling induced by trial 1. (1) Trial 1 induces 5HT release at the soma and synapse. (2) A TrkB ligand is released at the SN-MN synapse and binds pre-synaptic TrkB-like receptors. (3) A TrkB-dependent retrogradely transported, intracellular signal causes early-phase MAPK activation in SN somata at 45 min. (4) At the same time point, TrkB signaling increases *apc/ebp* mRNA expression.

(B) Molecular signaling induced by trial 2. (1) Trial 2 delivered at 45 min induces 5HT release at the soma and synapse. (2) A TGF-βr-II ligand is released at SN somata, where it binds somatic TGF-βr-II-like receptors, which is responsible for at least two functional outcomes. (3) 15 min after training, TGF-βr-II signaling interacts synergistically with the TrkB signaling cascade to prolong *apc/ebp* mRNA expression established by trial 1. (4) Additionally, TGF-βr-II signaling independently regulates late-phase MAPK activation in SN somata at a later time point (1 hr post-training).

activation of this complex alone can support LTF in the absence of CREB-mediated transcription (Bartsch et al., 2000; Lee et al., 2006). Previous results show that *apc/ebp* mRNA stabilization can be bi-directionally regulated by the AU-rich element (ARE) RNA binding proteins ApAUF1 and ApELAV (Lee et al., 2012; Yim et al., 2006). ApAUF1 binding to the 3′ UTR of *apc/ebp* induces the degradation of the transcript, and overexpression of ApAUF1 inhibits 5HT-induced LTF in SN-MN coculture (Lee et al., 2012). Conversely, ApELAV binds in the same ARE region, but, in contrast to ApAUF1, it stabilizes the transcript (Yim et al., 2006).

Interestingly, there is evidence that TGF-β can initiate stabilization of mRNAs via an RNA binding complex (Amara et al., 1995). For example, in cardiac fibroblasts, TGF-β1 treatment induced HuR (a member of the ELAV family) translocation from the nucleus to the cytoplasm, where it bound the ARE region of the 3′UTR in *tgfβ1* mRNA, therefore stabilizing the mRNA and increasing protein expression (Bai et al., 2012). These data suggest the intriguing possibility that trial 2 TGF-βr-II signaling may initiate a cascade that recruits ELAV family RNA binding proteins to prolong the expression of critical learning-related genes like *c/ebp*. Furthermore, other GF ligands could be induced and stabilized in the same way (including a TGF-β1-like ligand itself).

MAPK activation may also play a role in mRNA stabilization. Nerve growth factor (NGF) and epidermal growth factor (EGF) stimulation of PC12 cells stabilized m4 muscarinic acetylcholine receptor mRNA in a MEK-dependent (the upstream activator of MAPK) and protein synthesis-dependent manner (Lee and Malek, 1998). MAPK activation has also been implicated in the HuR binding and subsequent stabilization of mRNAs in hepatocytes (Yashiro et al., 2013) and non-small-cell lung carcinoma cells (Yang et al., 2004) and is required for the stabilization of GF ligand mRNAs in cardiomyoblasts (Miller et al., 2012). Additionally, ApC/EBP protein has been shown to require MAPK phosphorylation for its DNA-binding capability in addition to protecting it from proteasomal degradation (Yamamoto et al., 1999). Since both prolonged expression of *apc/ebp* (Figure 5B_3) and latephase MAPK activation (Figure 2A_3) is dependent on trial 2 TGF-βr-II signaling, it will be important to determine the role of TGF-βr-II-dependent MAPK signaling in the regulation of ApC/EBP mRNA and protein.

In conclusion, the results in this paper, taken collectively, emphasize the rich and complex interactions between multiple GF families, both in parallel and synergistically, during memory formation. These data therefore support the notion (Kopec and Carew, 2013) that GFs do not act in isolation but, rather, as a complex, spatiotemporally regulated molecular network that is engaged in the service of information storage within a neural circuit.

EXPERIMENTAL PROCEDURES

Ganglion Preparation

Pleural-pedal ganglia were dissected from anesthetized *Aplysia californica* (150–250 g, South Coast Bio-Marine). In a 1:1 solution of $MgCl_2$ and artificial sea water (ASW), the pleural SN cluster and SN-MN neuropil were exposed. For split-bath analyses, the desheathed ganglia were drawn over a plastic partition so that the SN somata (somatic compartment) and SN-MN synapses (synaptic compartment) were on different sides. The partition was sealed with Vaseline so that the molecular environment of the compartments could be manipulated independently of one another (Sherff and Carew, 1999). Ganglia were perfused with ASW for at least 1 hr to clear $MgCl_2$ prior to experimentation. Experimental and contralateral within-animal control ganglia both received GF chimera/vehicle treatment, while only the experimental ganglia received wo-trial TNS training.

Training and Drug Incubation for MAPK Analyses

The p9 tail nerve was inserted into a suction electrode, and two trains of electrical stimulation (1.5 s train: 30 V, 40 Hz, 5 ms pulses, inter-trial interval (ITI) = 45 min) were delivered. Ganglia were blocked with 0.1% BSA 5 min prior

to GF chimera (TrkB-Fc or TGF-βr-II-Fc, R&D Systems, 5 μg/ml) or vehicle (0.1% BSA in PBS) incubation to reduce nonspecific binding. The chimeras are polypeptides containing the extracellular portion of human TrkB or TGF-βr-II and were reconstituted at 100 and 50 μg/mL, respectively, in PBS with 0.1% BSA. Pilot experiments guided by prior use of TrkB receptor bodies in this system tested a range of concentrations (1–5 μg/ml) and determined that 5 μg/ml exerted a maximal inhibitory effect on MAPK activation (data not shown). Therefore, we used a final concentration of 5 μg/ml for all experiments. This is consistent with the final effective dose used in previous studies in our laboratory (Sharma et al., 2006). The GF requirement was tested during trial 1 or trial 2 by applying GF chimera/vehicle to the bath (with mixing) 10 min prior to the target trial (e.g., 10 min prior to trial 1 to block GF signaling during trial 1). When necessary, the drug was washed out with ASW 5 min prior to the end of the target time window (e.g., 5 min prior to trial 2). Effective pre-incubation and washout times were determined in pilot experiments (data not shown; see the overall strategy in Philips et al., 2013b). For split bath preparations, the drug was added 20 min prior to the target time window without mixing to limit perturbation (e.g., 20 min prior to trial 1 to test GF during trial 1). SN somata were collected either 45 min after trial 1 (early-phase MAPK activation) or 1 hr after training (late-phase MAPK activation) and prepared for western blot analysis.

Western Blotting

Samples were loaded onto 4%–12% Bis-Tris gels (Novex; Life Technologies) for electrophoresis and then transferred to nitrocellulose membranes for incubation with primary antibodies (total MAPK [p44/42 MAPK, catalog no. 4696] and phospho-specific MAPK [phospho p44/42 MAPK, catalog no. 4370; Cell Signaling Technology]) and secondary antibodies IRDye 800CW and IRDye 680RD, LI-COR Biosciences]). MAPK activation (via its phosphorylation) within each sample was assessed using the LI-COR Odyssey imaging system. Each phospho-specific band was normalized to the total MAPK band within the same sample, and normalized phospho-MAPK in the experimental ganglia was compared to within-animal control ganglia. Data are displayed as percent of normalized MAPK activation relative to control.

GF Stimulation and Immunocytochemistry

Pleural SN-L7 abdominal MN co-cultures (at least 5 days in vitro [d.i.v.]; Zhao et al., 2009) were incubated with human biotinylated TGF-β1 or BDNF for 30 min at 4°C (R&D Systems Fluorokine kits, 2:5). Control experiments were performed in parallel using control protein with equivalent biotinylation (soybean trypsin inhibitor, provided in R&D Systems kits). Co-cultures were then incubated with avidin-fluorescein (2:7, R&D Systems) for 30 min at 4°C. The co-cultures were fixed (4% paraformaldehyde in 30% sucrose PBS for 15 min) and mounted with Pro-Long Gold antifade reagent (Life Technologies). Images were collected using a Leica SP5 and prepared with ImageJ software.

Training and Drug Incubation for Gene Expression Analyses

Two-trial analog training was delivered (2 s train: 30 V, 40 Hz, 5 ms pulses, ITI = 45 min), and ganglia were blocked with BSA. The drug was applied 20 min prior to the target trial and, when necessary, was washed out 20 min prior to the end of the target time window. SN somata were collected at the experimental time point and prepared for quantitative PCR analysis.

RNA Isolation, cDNA Synthesis, and Quantitative PCR Analysis

RNA was isolated and purified using QIAGEN RNeasy mini kit columns (QIAGEN). Total RNA was normalized for pairs of experimental and control ganglia (~100 ng), and cDNA was synthesized using Superscript III CellsDirect cDNA synthesis reagents (Invitrogen, CellsDirect). Quantitative PCR was performed using a Roche LightCycler 480 and SybrGreen (Roche, 3 min at 95°C, 30 cycles of 10 s at 95°C, 20 s at 50°C, and 30 s at 72°C). The following primers (5 μM) were used: *apc/ebp*-F 5′-caccacctcactcccatctc-3′, *apc/ebp*-R 5′-ctgacgtctgcgagactttg-3′, *apuch*-F 5′-gaagacgaagccactcaacc-3′, *apuch*-R 5′-tgagatggagcctgtgtgtc-3′, *apkhc1*-F 5′-aaggaagctgtcaggcagag-3′, *apkhc1*-R 5′-gatggctgtaccacctcctc-3′, *apgapdh*-F 5′-ctctgagggtgctttgaagg-3′, and *apgapdh*-R 5′-gttgtcgttgagggcaattc-3′. The quantity of each gene (measured by Ct) was normalized to *apgapdh* within the same sample (ΔCt), and then normalized values were compared between experimental and within-animal control groups (ΔΔCt). Data are displayed as fold induction relative to the control.

Semi-intact Behavioral Preparation

Preparations were prepared as described previously (Sutton et al., 2001). Briefly, the cerebral and paired pleural-pedal ganglia from anesthetized *Aplysia* (250–400 g, South Coast Bio-Marine) were isolated surgically, leaving p9 and pleural-abdominal innervation to the tail and abdominal ganglia, respectively, intact. The tail and mantle were removed surgically, and the siphon artery was cannulated with Dow Corning silastic tubing (0.025 inner diameter [I.D.], Fisher Scientific) and perfused at ~5 ml/min, while the tail was perfused at ~0.5 ml/min via three 22-gauge needles. The tail and mantle were pinned to the chamber floor, while the ring ganglia with both pleural-pedal ganglia desheathed were pinned in an isolated CNS chamber. Both chambers were perfused continuously with seawater (Instant Ocean, 15°C). The p9 and pleural-abdominal nerves exited the CNS chamber through small slits that were sealed with Vaseline. Preparations were allowed to recover for at least 2 hr prior to baseline measurements.

Training and Drug Incubation for LTM Analyses

An average of three to four baseline T-SWR measurements were recorded by stimulating the medial posterior tip of the tail with a water jet (0.4 s, 45 psi, ITI = 15 min, Teledyne Water Pik). There were no significant differences in the pre-training baseline T-SWRs under any experimental condition (H = 0.7, df = 3, p = 0.9). The CNS was incubated with BSA, followed by drug 20 min prior to the target trial, which was washed out 15 min after the target trial. Two-trial LTM training (100 mA, 1.5 s, ITI = 45 min) was delivered medially to the anterior portion of the tail through a hand-held electrode. LTM for sensitization of the T-SWR was assessed by three to four tests (ITI = 15 min) 15–22 hr after training by an experimenter blind to the experimental condition (GF chimera versus vehicle). The data are displayed as the duration of T-SWR as a percent of the pre-training baseline.

Statistical Analyses

Because of the non-normality of the data, all data were analyzed using non-parametric statistics with GraphPad Prism. Within-group analyses were performed using Wilcoxon matched-pairs signed-rank tests. Specifically, MAPK activation and gene expression in experimental ganglia were compared with within-animal control ganglia, and the LTM for sensitization of T-SWR is compared relative to the pre-training baseline T-SWR within the same preparation. Between-group analyses (chimera versus vehicle) were performed using Mann-Whitney U tests. When appropriate, Kruskal-Wallis analyses were used to determine whether there was a difference among the groups, and significant results were followed by planned Wilcoxon or Mann-Whitney comparisons. Outliers greater than 2 SDs from the mean were excluded (Dixon and Tukey, 1968), resulting in the removal of ten data points (<4% of all data points, reported in the text). Data in all figures are depicted as median ± IQR. Significant within-group comparisons are displayed as asterisks above the summary data, and significant between-group comparisons are displayed as asterisks above a bar between the summary data.

AUTHOR CONTRIBUTIONS

A.M.K. conceived, designed, performed, and analyzed experiments. G.T.P. performed and analyzed experiments; and T.J.C. conceived and designed experiments. All authors wrote the paper.

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